

ATMOSPHERIC CO₂ AND O₃ ALTER THE FLOW OF ¹⁵N IN DEVELOPING FOREST ECOSYSTEMS

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Abstract. Anthropogenic O₃ and CO₂-induced declines in soil N availability could counteract greater plant growth in a CO₂-enriched atmosphere, thereby reducing net primary productivity (NPP) and the potential of terrestrial ecosystems to sequester anthropogenic CO₂. Presently, it is uncertain how increasing atmospheric CO₂ and O₃ will alter plant N demand and the acquisition of soil N by plants as well as the microbial supply of N from soil organic matter. To address this uncertainty, we initiated an ecosystem-level ¹⁵N tracer experiment at the Rhinelander (Wisconsin, USA) free air CO₂-O₃ enrichment (FACE) facility to understand how projected increases in atmospheric CO₂ and O₃ alter the distribution and flow of N in developing northern temperate forests. Tracer amounts of ¹⁵NH₄⁺ were applied to the forest floor of developing *Populus tremuloides* and *P. tremuloides*–*Betula papyrifera* communities that have been exposed to factorial CO₂ and O₃ treatments for seven years. One year after isotope addition, both forest communities exposed to elevated CO₂ obtained greater amounts of ¹⁵N (29%) and N (40%) from soil, despite no change in soil N availability or plant N-use efficiency. As such, elevated CO₂ increased the ability of plants to exploit soil for N, through the development of a larger root system. Conversely, elevated O₃ decreased the amount of ¹⁵N (~15%) and N (~29%) in both communities, a response resulting from lower rates of photosynthesis, decreases in growth, and smaller root systems that acquired less soil N. Neither CO₂ nor O₃ altered the amount of N or ¹⁵N recovery in the forest floor, microbial biomass, or soil organic matter. Moreover, we observed no interaction between CO₂ and O₃ on the amount of N or ¹⁵N in any ecosystem pool, suggesting that O₃ could exert a negative effect regardless of CO₂ concentration. In a CO₂-enriched atmosphere, greater belowground growth and a more thorough exploitation of soil for growth-limiting N is an important mechanism sustaining the enhancement of NPP in developing forests (0–8 years following establishment). However, as CO₂ accumulates in the Earth's atmosphere, future O₃ concentrations threaten to diminish the enhancement of plant growth, decrease plant N acquisition, and lessen the storage of anthropogenic C in temperate forests.

Key words: atmospheric CO₂; atmospheric O₃; *Betula papyrifera*; forest floor; microbial immobilization; N cycling; ¹⁵N; plant N uptake; *Populus tremuloides*; root system size; soil organic matter; Wisconsin, USA, developing forest.

INTRODUCTION

Will greater rates of terrestrial net primary productivity (NPP) be sustained as CO₂ accumulates in Earth's atmosphere? This question lies at the heart of our ability to predict the extent to which carbon (C) storage in terrestrial ecosystems might counterbalance anthropogenic CO₂ emissions over the next century (Houghton et al. 2001). Nevertheless, a clear answer to this simple question remains elusive, despite concerted efforts to understand the mechanisms by which atmospheric CO₂ might alter the C balance of terrestrial ecosystems (Luo et al. 2006). Although short-term exposure (i.e., years) to

elevated CO₂ increases the growth of many plants, much of the uncertainty surrounding this issue stems from potential interactions between atmospheric CO₂ and other environmental factors that may, over longer periods of time (i.e., decades and centuries), offset the positive effect of CO₂ on plant growth. Anthropogenic O₃ and CO₂-induced declines in soil nitrogen (N) availability (i.e., progressive N limitation sensu Luo et al. 2004) are two factors that could potentially diminish or eliminate greater plant growth in a CO₂-enriched atmosphere, thus constraining the long-term storage of anthropogenic CO₂ in terrestrial ecosystems.

Over the next century, O₃ in Earth's lower atmosphere is expected to attain concentrations (~65 nL/L) that will elicit phytotoxic effects across the Northern Hemisphere (Fowler et al. 1998, 1999). Recent analyses indicate that O₃ concentrations already present in this region (~40 to 70 nL/L) can reduce plant growth by 5–18%, with the

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largest reduction occurring in the belowground growth of temperate and boreal trees (Grantz et al. 2006). Boreal and temperate forests in the Northern Hemisphere are a globally important C sink (Ciais et al. 1995), and by 2100, ~60% of them could be exposed to damaging O₃ concentrations (Fowler et al. 1999). Because atmospheric CO₂ and O₃ will increase concomitantly over this period, it is plausible that future O₃ concentrations could lessen or nullify the effect of elevated CO₂ on plant growth, also diminishing the potential to store anthropogenic CO₂ in northern temperate forests.

Although anthropogenic O₃ can diminish the positive effect of atmospheric CO₂ on plant growth (King et al. 2005), it is not clear how these trace gases will combine to alter plant N demand and whether the supply of soil N can satisfy plant demand over the short and long term. Developing an understanding of this interaction is important, because plants in N-poor soil increase growth to a lesser extent under elevated CO₂ than those in N-rich soil (Zak et al. 2000, Reich et al. 2006). This observation implies that the supply of soil N will constrain plant growth enhancement, and hence ecosystem C storage, in a CO₂-enriched atmosphere. Furthermore, if elevated CO₂ alters plant detritus in such a way that decomposition slows and N availability to plants declines as some have argued (Field 1999, Luo et al. 2004, Hungate et al. 2006), then such a response could dampen future plant growth as atmospheric CO₂ accumulates. Nevertheless, empirical evidence for this argument is variable (Luo et al. 2006), and it is uncertain how exposure to both CO₂ and O₃ will alter the supply of N from soil organic matter as well as plant N demand.

In the Rhinelander (Wisconsin, USA) free air CO₂-O₃ enrichment (FACE) experiment, greater NPP has been sustained in developing forest communities exposed to elevated CO₂, but elevated O₃ has counteracted this response (King et al. 2005). Moreover, elevated CO₂ has increased gross N mineralization and microbial N immobilization to an equivalent extent, leading to more rapid rates of soil N cycling but no change in the supply of soil N to plants (Holmes et al. 2006). In contrast, elevated O₃ has reduced gross N mineralization, but it has not altered microbial N immobilization (Holmes et al. 2006), suggesting a decline in the supply of N to plants. Our primary objective was to quantify the extent to which changes in soil N cycling elicited by elevated CO₂ and O₃ have altered the flow of N among plants, soil microorganisms, and soil organic matter in developing northern temperate forests. Specifically, we set out to answer the following questions. Does exposure to elevated CO₂ alter plant N-use efficiency, and hence N demand? Do plants acquire equivalent amounts of soil N under ambient and elevated CO₂? Or, does greater belowground growth under elevated CO₂ increase the acquisition of soil N by plants? Do plants exposed to elevated O₃ acquire lower amounts of soil N due to a

decline in belowground growth (King et al. 2005), a decreased supply of soil N (Holmes et al. 2006), or a combination of both? To accomplish our objective and answer the aforementioned questions, we initiated an ecosystem-scale ¹⁵N-labeling experiment to trace the flow of mineralized NH₄⁺ into plants, soil microorganisms, and soil organic matter in young forests exposed to elevated atmospheric CO₂ and O₃.

METHODS

Research site

Our study was conducted at the Rhinelander FACE (free air CO₂-O₃ enrichment) facility located near Rhinelander, Wisconsin, USA (49°40.5' N, 89°37.5' E, 490 m elevation). Mean annual temperature is 4.9°C, mean annual precipitation totals 810 mm, and wet atmospheric-N deposition (from 1987 to 2005) averages $4.5 \pm 0.84 \text{ kg N·ha}^{-1}\cdot\text{yr}^{-1}$ (mean \pm SD). The experiment was established on a site with level topography and sandy-loam soil. Twelve 30-m-diameter FACE rings were constructed in a 32-ha field, with a minimum distance of 100 m between any two rings. Each ring was assigned to factorial CO₂ ($n = 2$) and O₃ ($n = 2$) treatments in a randomized complete-block ($n = 3$) design (Dickson et al. 2000). Target concentrations of CO₂ and O₃ were applied during daylight hours throughout the growing season (May through October). Elevated CO₂ was maintained near 560 $\mu\text{L/L}$ (200 $\mu\text{L/L}$ above ambient) and elevated O₃ was maintained at an average 50–60 nL/L (30–40 nL/L above ambient; Karnosky et al. 2005). Each ring was divided into east and west halves. The east half of each FACE ring was planted with five genotypes of trembling aspen (*Populus tremuloides* Michx.) that differ in O₃ sensitivity and CO₂ responsiveness. The west half of each ring was further divided into north and south quadrants. Northwest quadrants were planted with alternating aspen (genotype 216) and sugar maple (*Acer saccharum* Marsh.), and the southwest quadrant was planted with aspen (genotype 216) and paper birch (*Betula papyrifera* Marsh.) (details available online).⁵ Small individuals of each species (~10–15 cm tall) were planted in 1997 at a 1-m spacing, and, by 2003, canopy closure occurred in mixed aspen and aspen–birch sections. At that time, trees had attained heights of ~5 m.

At the initiation of the experiment, one ceramic-cup tension lysimeter was located at depths of 5, 30, and 125 cm in each plant community to sample soil water and quantify leaching losses. Soil-water samples were collected twice a month throughout the 2004 growing season. Prior to the first collection date and on each subsequent collection date, a tension equivalent to soil matric potential for that sampling interval (-0.05 to -0.06 MPa) was applied to each lysimeter (King et al. 2001). We used water from the lysimeters placed at 125

⁵ (http://aspenface.mtu.edu/ring_maps.htm)

cm in each FACE ring ($n=3$, one in each ring section) to estimate leaching losses of N and ^{15}N .

^{15}N tracer experiment

In June 2003 each 30-m-diameter FACE ring was labeled with tracer quantities of ^{15}N . Backpack sprayers were used to evenly dispense (0.034 L/m²) a dilute solution of $^{15}\text{NH}_4\text{Cl}$ (99.98% ^{15}N) over the forest floor. We applied $^{15}\text{NH}_4^+$ to follow the movement of NH_4^+ released during microbial mineralization into plants, the soil microbial community, and soil organic matter. The isotope was applied at the rate of 15 mg $^{15}\text{N}/\text{m}^2$, which represents ~3% of the inorganic N pool in mineral soil (0–10 cm depth). Immediately following application to the forest floor, 1.6 L/m² of water was applied to rinse the ^{15}N into mineral soil. Soil and plant samples were collected one week prior to and one year following (June 2004) isotope addition. This enabled us to determine the natural abundance of ^{15}N in plant and soil pools, and the extent to which they had been enriched by tracer ^{15}N . We allowed this tracer to flow among plant and soil pools for one year, and then we quantified the amount and distribution of ^{15}N and N in aspen and aspen–birch communities. Below, we describe the collection of plant, forest-floor, and mineral-soil samples as well as how we calculated the amount of tracer ^{15}N each ecosystem pool.

N and ^{15}N in aboveground plant pools.—To account for vertical variation in N and ^{15}N content within the overstory canopy, we sampled current-year shoots (20–25 cm in length) at four canopy levels: 75% to maximum canopy height, 50% to 75%, 25% to 50%, and below 25%. Canopy access was gained using a scaffold (7 m in height) located in each FACE ring that extended into each community type; canopy levels were identified using a height pole marked at 0.5-m intervals. At each canopy level, we collected one shoot sample from a randomly selected individual of each aspen genotype ($n=5$) in the aspen community. From randomly selected individuals in the aspen–birch community we collected two aspen shoot samples and two birch shoot samples. Shoot samples consisted of newly formed leaves, 4–6 mature leaves, and the small appending twigs; these plant tissues were frozen and transported to the laboratory for analysis. Following the collection of these samples, the vertical distribution of leaf area in aspen and aspen–birch communities was determined using a laser range finder (D. S. Ellsworth, *unpublished data*); leaf area profiles were measured in all FACE rings.

In the laboratory, leaf mass per area (LMA) was measured by removing 5–8 disks (0.5 cm in diameter) from each leaf. Leaves were separated from associated twigs; both were dried at 65°C, weighed, and ground to a fine powder. Leaves and twigs were analyzed for N concentration and $\delta^{15}\text{N}$ using a Delta plus isotope ratio mass spectrometer (Thermo-Finnigan, San Jose, Cal-

ifornia, USA) interfaced to a NC2500 elemental analyzer (CE Elantech, Lakewood, New Jersey, USA).

Using the diameter of each tree in aspen and aspen–birch communities, total leaf biomass was estimated using species-specific allometric equations derived from the destructive harvest of whole trees in 2004 (King et al. 2005). We then used the vertical distribution of leaf area and LMA determined for each canopy level to partition total leaf biomass, generated via allometric equations, among the four canopy levels. Total twig biomass was estimated by multiplying the twig-to-leaf ratio in each canopy level by leaf biomass. In each canopy level, leaf and shoot N content (g N/m²) were calculated as the product of tissue N concentration (in milligrams of N per gram, mg N/g) and biomass (in grams per square meter, g/m²). Additionally, we estimated ^{15}N in leaves and twigs in each canopy level as the product of atom percent (hereafter, atom %) excess ^{15}N , tissue N concentration, and biomass (in grams per square meter, g/m²). For each tissue, atom % excess ^{15}N was calculated as the difference between the atom % ^{15}N one year after isotope addition and the atom % ^{15}N prior to isotope addition. Values were summed across canopy height levels to generate total canopy N and ^{15}N content.

The N concentration and $\delta^{15}\text{N}$ of new stem tissue were determined by collecting a 15-mm diameter core, which extended from the stem surface into the newly formed bark and wood. This portion of the stem was easily penetrated by the sharpened coring device, unlike the harder wood produced from the previous year's growth (D. R. Zak, *personnel observation*). One stem core was removed from two individuals of each aspen genotype in the aspen community. In the aspen–birch community, stem samples were collected from two aspen and two birch, the same individuals used for canopy sampling. The N concentration and $\delta^{15}\text{N}$ of each stem sample was determined as described above. We estimated stem increment as the difference in stem biomass between 2004 and 2003, a value determined using the diameter of each tree in the aspen and aspen–birch communities and allometric equations for stem biomass (King et al. 2005).

Understory vegetation also was sampled prior to, and one year following, isotope addition (Bandeff et al. 2006). This enabled us to estimate the amount of N and ^{15}N contained in this portion of the aspen and aspen–birch communities. The aboveground biomass of understory vegetation was harvested from four 0.5-m² areas randomly located in each community. Harvested plants were sorted by species and analyzed for N and ^{15}N as described by Bandeff et al. (2006).

Recovery of ^{15}N (%) in overstory leaves, twigs, stem increment, and roots was the amount of ^{15}N in each pool (mg $^{15}\text{N}/\text{m}^2$), relative to the total amount we applied (15 mg $^{15}\text{N}/\text{m}^2$); recovery in understory vegetation was calculated in the same manner.

N and ^{15}N in root and soil pools.—At the time of canopy sampling, we collected forest floor and mineral-

soil samples to determine the distribution of ¹⁵N in these pools. We randomly located two points in each ring section to sample forest floor. A 26 × 26 cm sampling frame was placed on the forest floor, and Oi and Oe material was removed, placed on ice while in the field, and then frozen. This material was thawed at a later date, dried at 60°C, and analyzed for N and ¹⁵N.

In each plant community we also collected eight soil cores (5 cm in diameter and 20 cm deep) at previously established random locations. Soil samples consisted of Ap- and B-horizon material; they were composited within each community and immediately placed on ice. Composite soil samples were immediately processed to separate roots from mineral soil. Roots were initially removed by hand, placed in a polyethylene bag, and frozen. A subsample of the remaining soil was elutriated to remove the remaining fine roots (sensu Pregitzer et al. 2000). Roots initially removed by hand and those elutriated from the subsample were sorted into size classes for further analysis (<0.5 mm, 0.5–1.0 mm, >1.0–2.0 mm, and >2.0 mm). Roots in each size class were dried, ground, and separately analyzed for N and ¹⁵N using a Delta plus isotope-ratio mass spectrometer interfaced to a NC2500 elemental analyzer (Thermo Finnegan, San Jose, California, USA). We report root N and ¹⁵N as the summation of values across all size classes.

The remaining root-free soil was used to determine the distribution of N and ¹⁵N among mineral-soil pools. To accomplish that task, we used a sequential extraction method to separate inorganic N, dissolved organic N, microbial N and soil organic N (sensu Holmes et al. 2003). Extractable NH₄⁺, NO₃⁻, and dissolved organic nitrogen (DON; depth, 0–20 cm) were initially separated from microbial N and soil organic N. A 12-g (fresh mass) soil subsample from the composite collected in each community was placed in a 30-mL glass vial to which we added 20 mL of 2 mol/L KCl. The vials were capped, placed on a shaker for 20 minutes and centrifuged for 15 minutes at 800 rpm (136g). Particulate organic matter and suspended cells were removed from the KCl solution by passing them through the 0.45-μm filter; the resulting filtrate contained inorganic N and DON. The extraction was repeated with a second 20-mL aliquot of 2 mol/L KCl. The filter from each syringe was placed back into the vial containing the corresponding soil. Filtrates were stored at 4°C prior to analysis.

Ammonium-N and NO₃⁻-N were sequentially diffused from the KCl extracts onto acid traps in preparation for ¹⁵N analysis (Brooks et al. 1989). Following diffusion of inorganic N, we used alkaline persulfate digestion to convert DON into NO₃⁻, which was subsequently diffused onto an acid trap as described above. The acid traps containing NH₄⁺, NO₃⁻ and DON were analyzed for atom% ¹⁵N on a Finnigan Delta Plus isotope-ratio mass spectrometer with a Conflo II interface (Thermo Finnigan, San Jose, California, USA). Ammonium-N and NO₃⁻-N concentrations were

determined prior to diffusion using an OI Analytical Flow Solution 3000 continuous-flow analyzer (OI Analytical, College Station, Texas, USA). Following alkaline persulfate digestion, DON (measured as NO₃⁻) was measured using automated colorimetry as described above.

A second extraction step was performed to separate microbial N and soil organic N. Previously extracted soils and filters (containing microbial cells) were fumigated with CH₃Cl for 5 d in a vacuum desiccator. Residual CH₃Cl was removed by repeated vacuuming, and 20 mL of 0.25 mol/L K₂SO₄ was then added to each vial. Vials were capped, placed on a shaker 30 minutes, and centrifuged for 15 minutes at 800 rpm (136g). The supernatant was decanted into a 120-mL specimen cup. This extraction was repeated with an additional 20-mL aliquot of K₂SO₄, and the extracts were frozen until they were digested to determine microbial N. The soil samples remaining in the vials were dried to a constant mass at 60°C in a forced-air oven. The dried soils were transferred to grinding jars, pulverized with stainless steel pins (0.5 cm diameter × 13.8 cm length) in a roller mill (Model 755RMV [U.S. Stoneware, East Palestine, Ohio, USA]), and stored for analysis of organic N.

Microbial N within the K₂SO₄ extracts was determined by alkaline persulfate digestion (Cabrera and Beare 1993). Blanks and glycine standards were digested simultaneously with samples. A 3-mL aliquot of each digest was used to determine NO₃⁻-N concentration, as described above. Nitrate-N in the remaining digest was captured on acid traps during a 5-d diffusion with MgO and Devarda's alloy (Fisher Scientific, Fair Lawn, New Jersey, USA). The acid traps were analyzed for atom% ¹⁵N by isotope-ratio mass spectrometry. Soil organic-N concentration was measured using a CE Instruments NC2500 elemental analyzer (CE Elantech, Lakewood, New Jersey, USA), and atom% ¹⁵N was then determined by isotope-ratio mass spectrometry. Nitrogen concentration, atom% ¹⁵N, and bulk density were used to calculate recovery of ¹⁵N (in percentage, %) and estimate the N pool (in grams of N per square meter, g N/m²) in extractable NH₄⁺, extractable NO₃⁻, extractable DON, microbial N, and soil organic N.

Recovery of ¹⁵N (%) in forest-floor and soil pools was calculated as the amount of ¹⁵N in each pool (mg ¹⁵N/m²), relative to the total amount we applied to (15 mg ¹⁵N/m²) to forest floor and mineral soil.

Leaching of N and ¹⁵N.—We quantified the N concentration and δ¹⁵N of NH₄⁺, NO₃⁻, and DON in soil water collected at a depth of 125 cm in each FACE ring. Soil-water samples composited across plant communities and were processed using the diffusion procedure described above to separate NH₄⁺, NO₃⁻, and DON.

Statistical analyses

We used an ANOVA for a split-plot, randomized, complete-block design to determine whether CO₂, O₃ or

TABLE 1. The main effects of atmospheric CO₂ and O₃ on the biomass, nitrogen concentration, and nitrogen content for overstory and understory plants.

Ecosystem component	Atmospheric CO ₂					
	Biomass (g/m ²)		N concentration (mg N/g)		N content (g N/m ²)	
	-CO ₂	+CO ₂	-CO ₂	+CO ₂	-CO ₂	+CO ₂
Overstory	1099 ^a (240.5)	1644 ^b (315.7)			12.4 ^a (2.93)	18.0 ^b (3.93)
Leaves	421.9 ^a (78.9)	592.2 ^b (123.2)	21.6 ^a (1.91)	22.0 ^a (1.63)	9.1 ^a (2.29)	13.1 ^b (3.03)
New twigs	194.6 ^a (41.9)	289.1 ^b (41.3)	8.4 ^a (0.95)	8.5 ^a (0.52)	1.6 ^a (0.51)	2.2 ^b (0.42)
Stem increment	300.0 ^a (111.1)	452.0 ^b (156.3)	4.8 ^a (0.54)	4.5 ^a (0.50)	1.4 ^a (0.44)	2.0 ^b (0.66)
Roots	183.2 ^a (50.7)	291.1 ^b (55.6)	7.4 ^a (0.80)	6.7 ^a (0.99)	0.36 ^a (0.09)	0.54 ^b (0.24)
Understory	59.1 ^a (17.1)	53.0 ^a (31.5)	20.3 ^a (1.48)	21.4 ^a (2.00)	1.2 ^a (0.35)	1.0 ^a (0.75)
Total plant	1158 ^a (231.6)	1697 ^b (304.3)			13.6 ^a (2.75)	19.0 ^b (3.53)

Notes: There was no two-way interaction of community type with either CO₂ or O₃, nor was there a significant three-way interaction among these factors. Values are main-effect means with standard deviations in parentheses; within rows, main-effect means with the same superscript lowercase letter are not significantly different at $\alpha = 0.05$. Blank cells indicate results were not possible.

their interaction altered the biomass, N concentration, N content and recovery of ¹⁵N in plant or soil pools in the aspen and aspen–birch communities. We also inspected data for departures from normality and homogeneity of variance, and, when needed, we transformed data to meet these assumptions. Treatment and interaction means were compared using a Fisher's protected LSD procedure, and significance for all analyses was accepted at $\alpha = 0.05$.

RESULTS

Ecosystem N pools

Community type did not interact with atmospheric CO₂ or O₃ to influence the biomass, N concentration, or N content of any ecosystem pool, indicating that aspen and aspen–birch communities responded in the same manner to these gases. There also was no significant interaction between atmospheric CO₂ and O₃ on the biomass, N concentration, or N content of plant and soil pools.

However, atmospheric CO₂ and O₃ exerted significant main effects on plant biomass and N content (Table 1), but not on tissue N concentration. For example, elevated CO₂ increased overstory biomass by 46%; increases of similar magnitude also occurred in leaves, twigs, stem increment, and roots (Table 1). Although

elevated CO₂ did not increase the biomass of understory plants, it did significantly increase total plant biomass (overstory + understory) by 46% (Table 1). We observed no effect of atmospheric CO₂ (main effect) on forest-floor biomass or soil organic matter (Table 2). In contrast, elevated O₃ caused significant declines in the biomass of leaves (−25%), twigs (−17%), stem increment (−27%), and roots (−18%), which resulted in a significant overall decrease in overstory biomass (−23%; Table 1). Elevated O₃ did not influence the biomass of understory plants, nor did it alter forest floor or soil organic matter (Tables 1 and 2).

Elevated CO₂ did not alter the N concentration (in milligrams of N per gram, mg N/g) of any ecosystem pool (Tables 1 and 2), whereas elevated O₃ only reduced the N concentration of understory plants and soil extractable NH₄⁺ and NO₃[−] pools (Tables 1 and 2). Although these atmospheric gases exerted little influence on N concentration, they did exert significant main effects on the N content (g N/m²) of overstory trees, albeit in opposite directions. For example, the N content of overstory trees increased 45% under elevated CO₂, whereas elevated O₃ resulted in a 29% decline (Table 1). These are directional responses that can be observed in all components of overstory biomass. Because neither CO₂ nor O₃ altered the N concentration of leaves, twigs,

TABLE 2. Main effects of atmospheric CO₂ and O₃ on the biomass, nitrogen concentration, and nitrogen content of soil pools.

Ecosystem component	Biomass (g/m ²)		N concentration (µg N/g)		N content (g N/m ²)	
	-CO ₂	+CO ₂	-CO ₂	+CO ₂	-CO ₂	+CO ₂
Forest floor	659.8 ^a (163.3)	749.6 ^a (163.1)	12.691 ^a (1735)	11.956 ^a (2168)	8.41 ^a (1.79)	9.05 ^a (2.27)
Soil organic matter	5713 ^a (646.9)	5754 ^a (636.9)	909.2 ^a (75.41)	935.8 ^a (123.81)	181.8 ^a (15.1)	187.2 ^a (24.7)
Microbial biomass			7.1 ^a (1.36)	7.6 ^a (0.79)	1.41 ^a (0.27)	1.52 ^a (0.16)
Extractable NH ₄ ⁺			1.9 ^a (0.62)	1.8 ^a (0.67)	0.39 ^a (0.12)	0.36 ^a (0.13)
Extractable NO ₃ [−]			0.6 ^a (0.23)	0.4 ^a (0.27)	0.11 ^a (0.05)	0.09 ^a (0.05)
Extractable DON			1.5 ^a (0.43)	1.7 ^a (0.47)	0.30 ^a (0.09)	0.34 ^a (0.09)

Notes: There was no two-way interaction of community type with either CO₂ or O₃, nor was there a significant three-way interaction among these factors. Values are main-effect means with standard deviations in parentheses; within rows, main-effect means with the same superscript lowercase letter are not significantly different at $\alpha = 0.05$. Blank cells indicate that results were not possible.

TABLE 1. Extended.

Atmospheric O ₃					
Biomass (g/m ²)		N concentration (mg N/g)		N content (g N/m ²)	
-O ₃	+O ₃	-O ₃	+O ₃	-O ₃	+O ₃
1554 ^a (365.5)	1190 ^b (337.0)	22.8 ^a (1.33)	20.8 ^a (1.54)	17.7 ^a (4.04)	12.6 ^b (3.18)
592.2 ^a (143.4)	441.9 ^b (90.9)	8.7 ^a (0.71)	8.2 ^a (0.73)	13.1 ^a (3.11)	9.1 ^b (2.19)
265.6 ^a (55.3)	218.1 ^b (63.6)	4.6 ^a (0.49)	4.9 ^a (0.53)	2.1 ^a (0.56)	1.7 ^b (0.50)
435.2 ^a (127.7)	316.8 ^b (159.4)	7.4 ^a (1.03)	6.7 ^a (0.72)	1.9 ^a (0.51)	1.5 ^b (0.70)
260.9 ^a (70.2)	213.4 ^b (46.6)			0.55 ^a (0.24)	0.35 ^b (0.08)
43.2 ^a (10.8)	69.0 ^a (28.7)	21.7 ^a (1.77)	20.1 ^b (1.50)	0.8 ^a (0.31)	1.4 ^a (0.64)
1597 ^a (364.9)	1259 ^b (330.3)			18.6 ^a (3.88)	14.0 ^b (3.11)

wood, or roots, changes in overstory N content can be attributed to increases and decreases in overstory growth under elevated CO₂ and O₃, respectively. Despite their relatively large effect on overstory N content, CO₂ and O₃ (as main effects) did not alter the N content of understory plants, forest floor or mineral soil pools (Tables 1 and 2).

Recovery of ¹⁵N in ecosystem pools

Mean total recovery of ¹⁵N ranged from 78% to 79% and did not differ significantly from 100% across treatments (Table 3), indicating that we could account for the majority of isotope added to the aspen and aspen–birch communities. One year after application, the majority of added ¹⁵N resided in forest floor (~40%) and soil organic matter (~25%), with much smaller amounts in plant (~12%) and microbial (<1%) biomass (Table 3). The recovery of ¹⁵N in ecosystem pools was not influenced by any two-way or three-way interaction among community type, CO₂, or O₃.

However, as a main effect, atmospheric CO₂ significantly increased ¹⁵N recovery in the overstory trees, but it did not alter ¹⁵N recovery in any other ecosystem pool. Under elevated CO₂, canopy leaves contained 28% more ¹⁵N than did leaves grown at ambient CO₂ (Table 3). Although twigs, stem increment, and fine roots contained more ¹⁵N under elevated CO₂, those increases were not significant (Table 3). However, in combination, these trends amounted to a 35% increase in the amount

of ¹⁵N recovered in overstory trees growing under elevated CO₂, a response that was statistically significant (Table 3). The recovery of ¹⁵N in understory vegetation was relatively small (2–3%) and was not influenced by atmospheric CO₂. Elevated CO₂ increased ¹⁵N recovery by 29% in total plant biomass (overstory plus understory), but that increase was not significant. Atmospheric CO₂ had no effect on ¹⁵N recovery in forest floor or in any soil pool (Table 3).

As a main effect, atmospheric O₃ did not influence ¹⁵N recovery in any plant or soil pool (Table 3), with the exception of the small, but significantly greater, recovery of ¹⁵N in extractable NH₄⁺ and NO₃[−] pools (Table 3).

Concentrations of NH₄⁺ (0.04 ± 0.01 ug N/mL; mean ± SD), NO₃[−] (0.1 ± 0.08 ug N/mL) and DON (0.3 ± 0.22 ug N/mL) in soil water collected from lysimeters were routinely low in all treatment combinations, suggesting that leaching loss of N was minimal. For example, if we assume that all annual precipitation (74 cm in 2004) moved below the rooting zone, then leaching of NO₃[−] (0.06% of applied ¹⁵N) and DON (0.08% of applied ¹⁵N) can only account for a small proportion of the isotope we applied.

DISCUSSION

Greater plant growth in a CO₂-enriched atmosphere could increase the storage of anthropogenic C in terrestrial ecosystems, but projected O₃ concentrations and CO₂-induced declines in soil N availability could

TABLE 2. Extended.

Biomass (g/m ²)		N concentration (μg N/g)		N content (g N/m ²)	
-O ₃	+O ₃	-O ₃	+O ₃	-O ₃	+O ₃
758.3 ^a (155.5)	651.1 ^a (164.9)	12 534 ^a (1922)	12 113 ^a (2054)	9.52 ^a (1.83)	7.94 ^b (1.96)
5847 ^a (677.6)	5621 ^a (581.5)	958 ^a (102.8)	887 ^a (89.6)	191.6 ^a (20.6)	177.4 ^a (17.9)
		7.7 ^a (0.77)	7.0 ^a (1.32)	1.54 ^a (0.15)	1.40 ^a (0.26)
		2.4 ^a (0.27)	1.4 ^b (0.49)	0.47 ^a (0.05)	0.28 ^b (0.11)
		0.7 ^a (0.12)	0.3 ^b (0.18)	0.14 ^a (0.02)	0.06 ^b (0.04)
		1.6 ^a (0.36)	1.5 ^a (0.54)	0.33 ^a (0.07)	0.31 ^a (0.11)

TABLE 3. The main effect of atmospheric CO₂ and O₃ on the recovery of ¹⁵N in ecosystem pools one year following isotope addition.

Ecosystem component	¹⁵ N recovery (%)		¹⁵ N recovery (%)	
	-CO ₂	+CO ₂	-O ₃	+O ₃
Overstory	7.4 ^a (1.34)	10.0 ^b (2.08)	9.4 ^a (2.35)	8.0 ^a (1.80)
Leaves	5.0 ^a (0.94)	6.4 ^b (1.38)	6.1 ^a (1.2)	5.2 ^a (1.31)
New twigs	0.9 ^a (0.38)	1.26 ^a (0.38)	1.1 ^a (0.37)	1.1 ^a (0.45)
Stem increment	0.5 ^a (0.21)	0.6 ^a (0.30)	0.5 ^a (0.19)	0.5 ^a (0.32)
Roots	1.0 ^a (0.48)	1.8 ^a (1.46)	1.6 ^a (1.47)	1.2 ^a (0.68)
Understory	2.8 ^a (0.83)	3.2 ^a (2.08)	2.8 ^a (1.49)	3.2 ^a (1.68)
Total plant	10.2 ^a (1.66)	13.2 ^a (2.88)	12.2 ^a (2.87)	11.3 ^a (2.67)
Forest floor	40.1 ^a (12.36)	37.6 ^a (10.09)	42.0 ^a (11.10)	35.7 ^a (10.63)
Soil organic matter	20.1 ^a (8.34)	26.1 ^a (7.68)	22.4 ^a (7.96)	24.0 ^a (9.06)
Microbial biomass	0.8 ^a (0.58)	0.9 ^a (0.24)	0.9 ^a (0.54)	0.7 ^a (0.31)
Extractable NH ₄ ⁺	0.2 ^a (0.14)	0.2 ^a (0.11)	0.2 ^a (0.14)	0.1 ^a (0.10)
Extractable NO ₃ ⁻	0.0 ^a (0.01)	0.0 ^a (0.03)	0.0 ^a (0.02)	0.0 ^b (0.00)
Extractable DON	0.5 ^a (1.10)	0.1 ^a (0.10)	0.2 ^a (0.18)	0.4 ^a (1.11)
Total recovery	78.2 ^a (14.39)	78.7 ^a (11.48)	78.7 ^a (14.08)	79.2 ^a (11.90)

Notes: Data are means (with SD in parentheses). The recovery of isotope in each pool was calculated as the amount of ¹⁵N in that pool (mg ¹⁵N/m²), relative to the total amount applied to forest floor and soil (15 mg ¹⁵N/m²). There was no significant interaction between community type and either atmospheric CO₂ or O₃, nor was there a significant three-way interaction among these factors. Values are main-effect means with standard deviations in parentheses; within rows; main-effect means with the same superscript lowercase letter are not significantly different at $\alpha = 0.05$.

counteract this effect (Fowler et al. 1999, Luo et al. 2004). After eight years of CO₂ exposure, developing aspen and aspen–birch forests obtained greater amounts of soil N as well as ¹⁵N, despite no apparent increase in soil N availability (Holmes et al. 2006). Therefore, elevated CO₂ increased the ability of plants to exploit soil for this growth-limiting nutrient, which appears to result from a larger root system. This response has undoubtedly sustained higher rates of net primary productivity (NPP) in aspen and aspen–birch communities exposed to elevated CO₂, relative to those growing at the ambient atmospheric concentration (King et al. 2005). In contrast, elevated O₃ had an opposite effect, wherein plants exposed to this trace gas experience a decline in growth and acquired less soil N and ¹⁵N. Because CO₂ and O₃ did not interact to influence plant growth (King et al. 2005) or the amount of N and ¹⁵N in plant and soil pools, our results imply that elevated O₃ could reduce the ability of plants to grow and acquire N from soil as CO₂ increases in the atmosphere. The fact that plants exposed to elevated CO₂ obtained greater amounts of soil N indicates that progressive N limitation (sensu Luo et al. 2004) has not occurred in these developing forest communities—at least not yet.

Greater rates of plant growth under elevated CO₂ could be sustained by an increase in N-use efficiency (Gill et al. 2002, Hungate et al. 2006); however, several pieces of evidence indicate that elevated CO₂ did not increase the efficiency of N use (biomass produced per unit of N assimilated) in aspen and aspen–birch communities. First, elevated CO₂ has not altered biomass allocation in these communities or fine-root turnover (Pregitzer et al. 2000, King et al. 2005), indicating above- and belowground litter production under elevated CO₂ increases proportionally with plant biomass. If elevated CO₂ had increased plant N-use

efficiency, then such a response would be accompanied by a measurable decrease in the N concentration of some plant tissue. In contrast to this prediction, the N concentration of overstory leaves, twigs, stem increment and roots was unaltered by elevated CO₂; the same was true for the aboveground portion of understory plants. In our experiment, plants exposed to elevated CO₂ grew to a greater extent via enhanced rates of photosynthesis (Karnosky et al. 2003, King et al. 2005) and a greater exploration of soil for limiting resources (i.e., N as well as ¹⁵N), rather than an increase in plant N-use efficiency.

Our observations are consistent with those in young sweet gum (*Liquidambar styraciflua*) and loblolly pine (*Pinus taeda*) forests exposed to elevated CO₂ (Finzi et al. 2006, Norby and Iversen 2006); in both cases, elevated CO₂ increased the N content (as g N/m²) of overstory trees via greater belowground growth, with no or minimal change in plant N-use efficiency (Finzi et al. 2006, Norby and Iversen 2006). Moreover, elevated CO₂ has not altered soil N availability in our experiment as well as beneath sweet gum or loblolly pine in the experiments mentioned above (Zak et al. 2003, Finzi et al. 2006). If soil N availability has not increased under elevated CO₂, then how did plants growing under elevated CO₂ obtain more N from soil to sustain NPP?

In all three rapidly developing forests, greater rates of NPP under elevated CO₂ appear to have been sustained, in part, by a more thorough exploitation of soil by a larger root system (Matamala and Schlesinger 2000, Norby et al. 2004, King et al. 2005). In our experiment, it is unlikely that the increment of N plants acquired under elevated CO₂ resulted from greater specific root uptake (i.e., ug N·g_{root}⁻¹·s⁻¹), because elevated CO₂ does not alter the uptake kinetics in the fine roots of either aspen or birch (Rothstein et al. 2000; A. Friend, *unpublished data*). Rather, elevated CO₂ may have

enabled a larger root system to more effectively encounter and penetrate N-rich microsites, thereby facilitating foraging for this limiting resource (Johnson et al. 2006). Additional support for this idea comes from the positive relationship between annual N uptake and cumulative fine-root length in sweet gum, further suggesting that plants obtained greater amounts of soil N under elevated CO₂ by occupying soil to a greater extent (Norby et al. 2004). Despite these responses, greater soil occupancy will diminish through time as forests mature and fine roots exploit soil to a full extent. This point will be a critical juncture determining whether greater rates of NPP will be sustained in a CO₂-enriched atmosphere. If elevated CO₂ does not increase plant N-use efficiency or soil N supply when roots have fully occupied soil, then plant growth enhancement may be constrained by soil N availability at this point in development.

However, one does not need to invoke mechanisms creating a greater N supply under elevated CO₂ (e.g., greater N₂ fixation or organic-N uptake) to account for the additional N obtained by plants in our experiment. The additional N obtained by plants growing under elevated CO₂ was much less than the variability that exists in the supply of soil N to plants (i.e., net N mineralization). For example, plants growing under elevated CO₂ acquired ~0.7 g N·m⁻²·yr⁻¹ more than those growing under ambient CO₂. Net N mineralization in Lake States forests ranges from 5 to 12 g N·m⁻²·yr⁻¹ and coefficients of variation are 20 to 45% ($n = 59$ stands; Zak and Pregitzer 1990, Reich et al. 1997). Inasmuch, the additional N plants acquired under elevated CO₂ lies well within the bounds of variability for even the most N-poor forests in this region (e.g., 1 SD ranges from 1.0 to 2.2 g N·m⁻²·yr⁻¹). Although annual net N mineralization has not been measured in our experiment, evidence suggests this soil is not N poor. Rates of gross N mineralization and immobilization in Rhinelander (Wisconsin, USA) FACE soil are equivalent to those in Lakes States forests with relatively high net N mineralization (8–10 g N·m⁻²·yr⁻¹; Holmes and Zak 1999, Holmes et al. 2006). These higher rates and their associated variability further suggest that spatial variation in N supply can well account for the additional N obtained by a plant under elevated CO₂. Our argument implies that N loss via leaching should be greater under ambient CO₂; however, variation in plant water use between communities and treatments as well as variation in soil properties (e.g., thickness of a clay-rich subsurface horizon) make it difficult to resolve whether N leaching differs between ambient and elevated CO₂.

Although substantial amounts (~60%) of ¹⁵N were recovered in forest floor and soil organic matter after one year, we have no evidence that greater litter production under elevated CO₂ has accelerated the net microbial immobilization of N into soil organic matter. Such a response would presage a decline in soil N

availability, and this clearly has not occurred in our experiment. For example, elevated CO₂ did not alter the recovery of ¹⁵N in forest floor or soil organic matter, nor did it alter the N content of any soil pool. If elevated CO₂ had increased the net microbial immobilization into forest floor or soil organic matter, then one would expect to find greater amounts of ¹⁵N in these pools, which is inconsistent with our observations. Rather, the responses we observed likely arose due to an equivalent increase in gross N mineralization and microbial immobilization elicited by elevated CO₂ (Holmes et al. 2006). Our data clearly demonstrate that greater plant growth under elevated CO₂ and the subsequent production of litter have not fostered a decline in soil N availability over the first eight years of our experiment.

Our observations contrast with a greater net immobilization of ¹⁵N into the forest floor of scrub oak woodland exposed to elevated CO₂ (Hungate et al. 2006), a community which sprouted from an established root system following fire. In that ecosystem, elevated CO₂ also initially increased the acquisition of soil N by plants, but this response diminished after seven years of exposure. In combination with a greater incorporation of N into forest floor, this decline in plant N acquisition was reported to indicate the onset of progressive N limitation (Hungate et al. 2006). These responses differ from those in our experiment and may be due to differences in forest development, the extent to which a newly developing or preexisting root system has exploited soil, or native soil N availability. Regardless of this disparity, elevated CO₂ has not elicited greater rates of microbial N immobilization, a decline in soil N availability, or the onset of progressive N limitation in our experiment (Holmes et al. 2006); this also appears to be the case in other young, developing forests dominated by sweet gum and loblolly pine (Zak et al. 2003, Finzi et al. 2006, Norby and Iversen 2006).

In contrast, elevated O₃ damaged leaves, which led to declines in photosynthesis, above- and belowground growth, plant N demand, and the acquisition of soil N. Ozone also had no effect on plant N-use efficiency, evidenced by equivalent tissue-N concentrations in plants exposed to ambient and elevated concentrations. It is possible that aspen and aspen–birch communities exposed to elevated O₃ obtained lower amounts of soil N due to an overall decline in growth. We are unable to discern if concomitant declines in soil N availability (Holmes et al. 2006) partially contributed to this response, but it appears unlikely given the overall decline in growth, plant N demand, and acquisition of soil N elicited by elevated O₃. Taken together, these observations indicate that elevated O₃ has reduced the rate at which N cycles among plant and soil pools, an effect directly opposed to that of elevated atmospheric CO₂.

Notwithstanding a reduction in forest-floor mass and N content, diminished plant growth in an O₃-enriched atmosphere was insufficient to alter the N content or flow of ¹⁵N among soil pools. This result was somewhat

surprising, because microbial communities beneath plants exposed to elevated concentrations of both O₃ and CO₂ can metabolize greater amounts of recent photosynthate than those exposed elevated to CO₂ alone (Loya et al. 2003). These presumably labile substrates should increase rates of microbial immobilization, and subsequently the incorporation of N into forest floor and soil organic matter. However, such a response is inconsistent with the equivalent amounts of ¹⁵N recovered in forest floor under ambient and elevated O₃; soil organic matter exhibited the identical response. The fact that elevated O₃ did not influence ¹⁵N recovery in either of these pools implies that it also did not influence rates of microbial N immobilization, at least over the eight-year duration of our experiment. This reasoning is further supported by a recent analysis, in which elevated O₃ had no effect on microbial N immobilization (Holmes et al. 2006). It also appears that lower rates of gross N mineralization (~16%; Holmes et al. 2006) under elevated O₃ were insufficient to alter ¹⁵N recovery in soil organic matter. Overall, the most apparent influence elevated O₃ had on soil pools was to decrease leaf-litter production, thereby lowering the mass and N content of forest floor.

In these developing aspen and aspen–birch, higher rates of NPP have been sustained by a more thorough exploitation of soil for N, evidenced by the greater amount of N and ¹⁵N obtained by plants growing in a CO₂-enriched atmosphere. This response occurred despite no apparent change in soil N availability or plant N-use efficiency. We conclude that progressive N limitation is presently not a factor governing plant growth response to elevated CO₂ in these young, developing forest communities. On the other hand, elevated O₃ exerted a substantial negative influence on plant growth, plant N demand, and the amount of N plants obtained from soil. The absence of a significant interaction between atmospheric CO₂ and O₃ on plant growth or N content suggests that O₃ could elicit a negative effect regardless of the atmospheric CO₂ concentration. However, such a response might be moderated by plant species or genotypes that are insensitive to the effects of O₃ (Coleman et al. 1995a, b). Because damaging O₃ concentrations are expected to occur across broad regions in the Northern Hemisphere (Fowler et al. 1998, 1999), our observations indicate that future O₃ concentrations have the potential to diminish the enhancement of plant growth, decrease plant N acquisition, and lessen the storage of anthropogenic C in temperate forests as CO₂ accumulates in Earth's atmosphere.

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